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Oxygen Control of the *Bradyrhizobium japonicum hemA* Gene

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The *hemA* gene of *Bradyrhizobium japonicum*, which encodes the first enzyme in the heme biosynthetic pathway, is regulated by oxygen. Up to ninefold induction of β-galactosidase activity is seen when cultures of *B. japonicum* containing either a plasmid-encoded or a chromosomally integrated *hemA-lacZ* fusion are shifted to restricted aeration. The oxygen effect is mediated via the FixLJ two-component regulatory system, which regulates the expression of a number of genes involved in the nitrogen fixation process in response to low-oxygen conditions; oxygen induction is lost when the *hemA-lacZ* fusion is expressed in strains of *B. japonicum* carrying mutations in *fixL* or *fixJ*. The *B. japonicum hemA* promoter region contains a sequence identical to the *Escherichia coli* Fnr binding site (positions −46 to −33 relative to the *hemA* transcription start site). Fnr is a regulatory protein necessary for the oxygen-regulated expression of anaerobic respiratory genes. Activity of a *hemA-lacZ* fusion construct in which the Fnr box-like sequence was replaced with a *Bgl*II site is not induced in *B. japonicum* cultures grown under restricted aeration. The *fnr* homolog *fixK* is FixLJ dependent. Collectively, these data suggest a role for the rhizobial Fnr-like protein, FixK, in the regulation of *hemA*. Furthermore, the coregulation of *hemA* with symbiotically important genes via FixLJ is consistent with the idea that *hemA* is required in the nodule as well as under free-living conditions.

Bradyrhizobium japonicum can live free in the soil or it can enter into a symbiotic relationship with soybean plants that results in the development of nodules, specialized structures on the plant's roots within which the bacteria reside and fix nitrogen. One prominent feature of these nodules is that they are microaerobic (23), which raises the question of how the symbiotic bacteria, called bacteroids, can operate at very low oxygen levels while meeting the high-energy demands of nitrogen fixation. Adaptations by both the plant and the bacterial partners provide the solution to this seeming dilemma, as these adaptations allow efficient bacteroid respiration to take place in nodules. Briefly, bacteroids utilize a terminal oxidase complex different from that used by free-living cells (4); this complex functions at the low-oxygen tensions found in nodules and is most likely encoded by the *fixNOPQ* operon, which has recently been shown to be induced under microaerobic conditions and to be essential for the symbiosis (43). In addition, the cytochrome content of bacteroids is three times that of cultured cells (48), presumably to facilitate the generation of ATP for nitrogen fixation. On the plant side, cells infected with bacteroids contain leghemoglobin, a nodule-specific protein that facilitates oxygen diffusion to the respiring bacteroids (4). Leghemoglobin is absolutely essential for the symbiosis.

The elevated levels of cytochromes in bacteroids and the presence of leghemoglobin in nodule cells translate into an increased demand for heme in nodules. It has been suggested that the bacteroid provides the heme for leghemoglobin in addition to synthesizing heme for its own needs. Unfortunately, the origin of the heme moiety of leghemoglobin remains unresolved, despite studies with bacterial mutants blocked at three different points in the heme biosynthetic pathway. These mutants either could be rescued by the plant (i.e., *B. japonicum* mutants blocked at the first step in the heme biosynthetic pathway [19]) or were blocked very early in nodule development, long before the apoleghemoglobin protein would be synthesized (i.e., *B. japonicum* mutants blocked at the second [7] or sixth [16] step in the heme biosynthetic pathway).

We are interested specifically in *hemA*, the gene encoding Δ-aminolevulinic acid synthase (ALAS), which catalyzes the first step in the heme pathway. One control on *hemA* expression is oxygen. *B. japonicum* cells grown in fermentors bubbled under controlled oxygen tensions ranging from 5 to 0.2% oxygen have 6- to 10-fold more *hemA* mRNA than aerobically grown cells (53). In nodules, this oxygen control is likely to occur via the same two-step regulatory cascade already shown to control *nif* and *fix* genes in various rhizobial species. These oxygen-regulated genes are activated via the FixLJ two-component regulatory system (1, 3, 10, 25). FixL (17, 34), the sensor, is a hemoprotein that under low-oxygen conditions transmits a signal via phosphorylation to the response regulator, FixJ (20, 41), which in turn activates specific *nif* and *fix* genes (30, 45, 59, 60). Several FixLJ-regulated gene products with similarity to the *Escherichia coli* Fnr protein. Fnr controls the expression of target genes in response to anoxia by binding to a specific promoter motif termed an Fnr box (for reviews, see reference 21, 54, and 55). Rhizobial Fnr-like proteins include FixK from *Rhizobium meliloti* (7), $FixK_1$ and $FixK_2$ from *B. japonicum* (2, 3), and FnrN from *Rhizobium leguminosarum* biovar viciae (8). As might be expected, Fnr boxes have been noted upstream of a number of rhizobial genes, including the *B. japonicum hemA* gene (15, 38, 40, 44).

In this study, we used *hemA-lacZ* fusions to further investigate the response of *hemA* to restricted aeration. Deletion of the Fnr box-like sequence upstream of the *hemA* gene abolishes induction under restricted aeration, suggesting the involvement of the rhizobial Fnr homolog FixK in the regulation of *hemA*. Furthermore, we show that activation of *hemA* is FixLJ dependent. The coordinate regulation of *hemA* with the synthesis of symbiotically relevant proteins is consistent with an important role for *hemA* in the nitrogen fixation process.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this work are listed in Table 1. *B. japonicum* strains were propagated in AG medium (46) or in minimal medium (19). Tetracycline was used at 100 μg/ml. Plasmid

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
E. coli CJ236	Host for in vitro mutagenesis; Cm^r *dut ung thi relA*	31
B. japonicum		
USDA 110	USDA 3I1b110	19
2925	*hemA-lacZ* chromosomal insertion	42
7404	Spc^r Km^r Tc^r; *fixL*::*aph*[a]	1
7361	Spc^r Km^r Tc^r; *fixJ*::*aph*	1
pEC1	*B. japonicum hemA-lacZ* fusion from pBJ289 cloned into the *Sal*I site of pBluescript SK$^-$	This study
pKP21	*Hin*dIII-*Eco*RI fragment of pEC1 cloned into pBluescript KS$^-$. This carries the region upstream of the *hemA-lacZ* fusion junction	This study
pBluescript SK$^-$	Ap^r	Stratagene
pBluescript KS$^-$	Ap^r	Stratagene
pRK2013	Km^r helper plasmid for mobilization	14
p3-76	pIBI76 polylinker (IBI) cloned into the *Eco*RI site of pLAFR3	M. Mindrinos
pBJ289	*B. japonicum hemA-lacZ* fusion cloned into pRK290 (11)	42
pEC100	*B. japonicum hemA-lacZ* fragment cloned into *Bam*HI-*Kpn*I sites of p3-76	This study
pEC100ΔFBX	pEC100 Fnr box deletion (−46 to −33)	This study
p205-3-76	*hemA-lacZ* transcriptional fusion	This study

[a] Spc^r, resistance to spectinomycin; Km^r, resistance to kanamycin; Tc^r, resistance to tetracycline; *aph*, the gene encoding kanamycin resistance.

constructs were transformed into *B. japonicum* by either electroporation (20) or triparental mating (19).

Oxygen regulation of *B. japonicum*. *B. japonicum* cultures were grown to mid-log phase in AG medium. The cultures were diluted 1:100 into minimal medium containing 0.2% xylose. Because iron has a positive effect on *hemA* expression (42), no iron was added to the minimal medium to allow for a basal level of *hemA* expression. The cultures were allowed to grow to mid-log phase and split in half; half remained in the original flask, and half was transferred to a sterile screw-cap test tube filled to 1 cm from the top to limit oxygen availability. The cultures were incubated, with the flasks shaken and the test tubes on a rotary wheel. Similar protocols have been used to study oxygen effects on expression of other *B. japonicum* genes (3, 31). For the time course experiment whose results are shown in Fig. 1, an aliquot of culture was removed and frozen for the zero time point. The rest of the culture was then split in half, and restricted-aeration conditions were maintained for either 12, 24, or 48 h. Although we did see induction of β-galactosidase activity after 12 and 24 h, for the rest of the restricted-aeration experiments we chose to use a 48-h induction period. The cultures were frozen for later assay of β-galactosidase activity (37) and determination of protein concentration. Each culture was assayed in triplicate. β-Galactosidase specific activity is calculated as follows: $1{,}000 \times [A_{420}/t \times v \times$ mg of protein per ml] where t is the time of reaction in minutes and v is the reaction volume in milliliters. Protein concentration was determined by using the BCA protein assay according to the manufacturer's instructions (Pierce, Rockford, Ill.). Iron induction of *hemA-lacZ* fusion activity was performed as previously described (42).

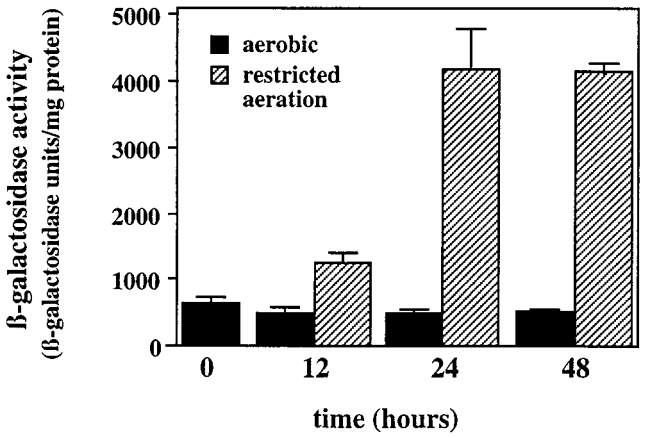

FIG. 1. Time course of expression of the *hemA-lacZ* fusion construct pEC100 in *B. japonicum* 110 under conditions of restricted aeration. Cultures of *B. japonicum* were frozen 12, 24, and 48 h after transfer to restricted aeration and at the time of transfer (0 h) for later assay of β-galactosidase activity as described in Materials and Methods.

Construction of the *hemA-lacZ* fusions. pEC100 differs from the *hemA-lacZ* fusion pBJ289 used in our previous study (42) by the inclusion of a polylinker; both plasmids are pLAFR1 derivatives. The polylinker was added to facilitate the creation of deletions and other constructs made with the *hemA-lacZ* fusion. To create pEC100, pBJ289 was digested with *Sal*I and the fragment containing the *hemA-lacZ* fusion was inserted into the *Sal*I site of pBluescript SK$^-$ (Stratagene, La Jolla, Calif.) to create pEC1. The gene fusion was then removed from pEC1 by digestion with *Bam*HI and *Kpn*I and was inserted into the same sites in the polylinker of vector p3-76 (the IBI 76 polylinker [IBI, New Haven, Conn.] subcloned into the polylinker of pLAFR3 [57]). The transcriptional fusion construct p205-3-76 contains a *hemA-lacZ* transcriptional fusion which differs from the translational fusion by replacement of the translational fusion junction of pEC100 with a fragment from the transcriptional fusion vector pRS415 (52). This fragment contains stop codons in all three reading frames followed by the native Shine-Dalgarno sequence and the start codon for the *lacZ* gene. *B. japonicum* 2925 carries a chromosomally encoded *hemA-lacZ* translational fusion constructed so that the fusion is integrated directly upstream of the chromosomal *hemA* gene and hence is contiguous with the region normally upstream of the endogenous gene (42).

Deletions upstream of the *hemA-lacZ* fusion coding region. The plasmid pEC1 (Table 1), containing the *hemA-lacZ* fusion and upstream flanking DNA to position −345, was digested with *Hin*dIII and *Pst*I before digestion with exonuclease III and S1 nuclease (New England Biolabs, Beverly, Mass.) to generate upstream deletion clones. Deletions were verified by DNA sequencing with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and inserted into the broad-host-range vector p3-76 (Table 1) at the *Bam*HI and *Kpn*I sites in the vector polylinker. The 14-bp Fnr box-like sequence, 5′-TTGATCGGGAT CAA-3′, from positions −46 to −33, was replaced with the 6-bp sequence 5′-AGATCT-3′, encoding a *Bgl*II site, via in vitro mutagenesis (31) using the pKP21 construct as a template. The deletion construct was confirmed by restriction digestion with *Bgl*II and by DNA sequencing using the DyeDeoxy Terminator Cycle Sequencing kit with the ABI sequencing system (Applied Biosystems, Foster City, Calif.). The *Hin*dIII-*Eco*RI fragment carrying the deletion was then subcloned back into pEC100, replacing the fragment which carried the wild-type sequence; this clone was designated pEC100ΔFBX.

RESULTS AND DISCUSSION

Oxygen regulation of *hemA*. The role of oxygen in the regulation of the *B. japonicum hemA* gene was investigated by comparing the activities of *hemA-lacZ* fusions in cultures of *B. japonicum* incubated aerobically with those of cultures incubated under restricted aeration. A time course experiment using cells carrying the translational fusion construct pEC100 demonstrated that cells incubated for 12 h under restricted aeration had approximately threefold more β-galactosidase activity than aerobically incubated cells and that cells incubated for 24 or 48 h under restricted aeration had approximately

TABLE 2. Expression of the *hemA-lacZ* fusion in *B. japonicum* grown under conditions of restricted aeration

Strain	Description	β-Galactosidase activity[a] (SE)		Fold induction, without O_2/ with O_2 (SE)	n[c]
		With O_2[b]	Without O_2[b]		
pEC100/110	Translational fusion	633 (73)	3,309 (304)	6.4 (1.2)	11
pEC100ΔFBX/110	Fnr box deletion	481 (67)	673 (67)	1.5 (0.2)	8
2925	Chromosomally integrated fusion	443 (107)	1,973 (390)	5.6 (1.8)	8
p205-3-76/110	Transcriptional fusion	2,175 (192)	9,815 (1,102)	4.6 (0.6)	6
pEC100-55/110	Deletion to position −55	941 (41)	3,585 (381)	3.9 (0.6)	4

[a] Expressed as β-galactosidase units per milligram of protein.
[b] Cultures of *B. japonicum* were frozen 48 h after transfer to restricted aeration for future measurement of β-galactosidase activity as described in Materials and Methods.
[c] n = number of independent assays.

ninefold more activity than aerobically incubated cells (Fig. 1). All three *hemA-lacZ* constructs showed more β-galactosidase expression when incubated under restricted aeration than when incubated aerobically (Table 2). The level of β-galactosidase activity under restricted aeration was 4.6-fold higher for the transcriptional fusion construct p205-3-76/110, 6.4-fold higher for the translational fusion construct pEC100/110, and 5.6-fold higher for strain 2925 (translational fusion integrated into the chromosome) than for cultures incubated for 48 h under aerobic conditions. Because the three constructs showed similar increases in expression when cells were incubated under restricted aeration, we concluded that the effect we were seeing was occurring at the level of transcription and was not influenced by whether the construct was plasmid borne or carried on the chromosome. We also conclude that the 6- to 10-fold increase in steady-state levels of *B. japonicum hemA* mRNA previously reported by Somerville and Chelm (53) was due to increased transcription of the *hemA* gene when cells where incubated under low levels of oxygen.

In contrast to our results, Kim et al. (29) did not observe induction of a plasmid-encoded *hemA-lacZ* fusion in *B. japonicum* 12 h after a shift to lower-oxygen conditions; after 12 h we did see induction, but it was only a third of that seen at 24 or 48 h (Fig. 1). In another study, Kim and Maier (28) monitored the activity of the same *hemA-lacZ* fusion for 60 h and still did not see induction under lower-oxygen conditions. Because both of these studies were performed with *B. japonicum* cultures growing autotrophically with CO_2 as a carbon source, the difference between their studies and ours may be attributable to the respiratory status of the cells. *hemA* expression is regulated by multiple factors, including heme (41) and iron (42); a response to lowered oxygen levels may not become apparent until heme levels change in response to an altered demand for electron transport components. We set up our experiment to minimize the effects of cell growth and iron availability. Cells were incubated in a minimal medium to which no iron was added. Initial and final protein concentrations of cultures incubated aerobically or under restricted aeration were always within twofold of one another, suggesting that differential growth is not responsible for the observed increases in β-galactosidase expression. That β-galactosidase activity remained constant in aerobically incubated cells over the duration of the experiment while β-galactosidase activity increased dramatically in cells incubated under restricted aeration suggests that restricted aeration was responsible for the observed effect.

Deletion of the Fnr box-like sequence abolishes oxygen regulation of *hemA*. Deletion analysis showed that the oxygen regulatory region must lie downstream of position −55 relative to the start of transcription (Table 2). A region of sequence starting at position −46 relative to the transcriptional start site exactly matches the consensus binding site for *E. coli* Fnr, $TTGATN_4ATCAA$ (38, 40, 44, 54, 55). Furthermore, most Fnr-dependent promoters in *E. coli* have Fnr boxes which are centered about 40 bp upstream of the transcriptional start site (13). Therefore, both the sequence and the location of the *hemA* Fnr box-like sequence suggested that it is a potential *cis*-acting oxygen regulatory site in *B. japonicum*. An internal deletion of the Fnr box-like sequence was created in the plasmidborne *hemA-lacZ* fusion construct by replacement of the 14 nucleotides constituting the Fnr box-like sequence with a 6-bp *Bgl*II site. β-Galactosidase activity was no longer induced when cells carrying this deletion construct on a plasmid were grown under restricted aeration (Table 2), indicating that the Fnr box-like sequence is needed for oxygen regulation. In order to demonstrate that deletion of the Fnr box-like sequence affects oxygen regulation specifically, the Fnr box deletion construct was tested for iron inducibility. Previous experiments in our laboratory have determined that expression of *hemA* is induced by iron (42). Cells carrying the deletion construct behaved identically to cells carrying the wild-type fusion, showing an approximately threefold induction by iron (Table 3). Thus, the Fnr box-like sequence appears to be specific for the oxygen effect.

Fnr box-like sequences. Table 4 presents Fnr box-like sequences from genes involved in heme synthesis or nitrogen fixation from various rhizobial and rhodobacter species. A consensus based on the 21 genes listed differs only slightly from the "*Rhizobium* anaerobox" arrived at by Colonna-Romano et al. (8) from a comparison of nine rhizobial genes. Both in vitro

TABLE 3. Effects of *cis* and *trans* oxygen regulators on iron induction of *hemA*

Strain	Relevant genotype	β-Galactosidase activity[a] (SE)		Fold induction, with Fe/ without Fe	n[c]
		With Fe[b]	Without Fe[b]		
pEC100/110	wt[d]	2,097 (177)	755 (68)	3.3 (0.3)	31
pEC100ΔFBX/110	ΔFBX	1,545 (52)	489 (75)	3.6 (0.6)	6
pBJ289/110	wt	4,258 (466)	1,409 (450)	3.4 (0.7)	3
pBJ289/7361	*fixJ*	1,613 (222)	541 (170)	3.0 (0.2)	8
pBJ289/7404	*fixL*	2,306 (388)	524 (69)	4.5 (0.5)	5

[a] Expressed as β-galactosidase units per milligram of protein.
[b] Cultures of *B. japonicum* were grown under iron-deficient growth conditions to mid-log phase in minimal medium. The culture was then split, and half the culture was supplemented with 10 μM iron. After 24 h, the cultures were stored frozen for future measurement of β-galactosidase activity.
[c] n = number of independent assays.
[d] wt, wild type.

TABLE 4. Comparison of Fnr box-like sequences from *Agrobacterium radiobacter* and various nitrogen-fixing bacteria

Gene or sequence	Species	Fnr box-like sequence[a]	Location[b]	Reference
hemA	*B. japonicum*	ttctTTGATcgggATCAAgttt	−46 tc	36
hemA	*R. meliloti*	ttgcTTGACttcgATCGAtgtt	−34 P1, −273 P2	33
hemA	*Agrobacterium radiobacter*	ttttTTGATgtgaATCAAtttt	−48 P1, −145 P2	12
hemA	*Rhodobacter sphaeroides*	ggatTTGATccttATCAAggcc	−6 tc	39
hemB	*B. japonicum*	aagaGTGATggcgATCAAatac	−3 tl	7
fixG	*B. japonicum*	ccgtTTGAGctggATCAAcgga	−71 tl	43
fixG	*R. meliloti*	agacTTGACgcagATCAAggtg	−79 tl	24
fixJ	*B. japonicum*	acgaTTGAGccaaGTCAAggcc	−47 tc	1
fixK	*B. japonicum*	cgagTTGATctcgCGCAAtgcg	−487 tc	58
fixL	*R. meliloti*	tacaTTGATcacgGTCAAtact	−171 tl	10
fixN	*R. meliloti*	tcctTTGACttgtATCAAggtg	−47 tc	35
fixN	*A. caulinodans*	tatcTTGATttcaATCAAttcc	−70 tl	43
fixN	*B. japonicum*	gcacTTGATctggATCAAggtg	−106 tl	5
fnrN	*R. meliloti*	cgctTTGATctagATCAAacag	−133 tl	8
fnrN	*R. leguminosarum* biovar viciae	gaaaTTGATaatcCTCAAgcgg	−101 tl	8
coxS1	*R. leguminosarum* biovar viciae	tttaTTGATccagATCAAagtg	−121 tl	51
anfK	*Rhodobacter sphaeroides*	cgccTCGATccagATCAAagcc	+81 tl	50
nifA	*Rhodobacter capsulatus*	aaatTTGATccagATCAAagcc	−147 tl	38
nifH	*A. caulinodans*	tcggTTGATggagAGCAAcatg	−15 tl	32
nodD	*Rhizobium* sp. strain BTAi1 *R. leguminosarum* biovar viciae	ggccTTGATattgATCAAgttc	−98 tl	56
E. coli consensus[c]		nannTTGATnnanATCAAtnnn		54
Nitrogen fixation[d] consensus		nnnnTTGATCnnGATCAAngnn		This study

[a] Uppercase letters denote the region of dyad symmetry from the *E. coli* consensus sequence.
[b] Location of the first T in the region of dyad symmetry relative to the transcriptional start site (tc) or the translational start site (tl). P, promoter.
[c] Uppercase letters denote the region of dyad symmetry, and lowercase letters denote other well-conserved nucleotides. n, any nucleotide.
[d] Uppercase letters indicate conserved nucleotides (>70% of the sequences contain this nucleotide), and lowercase letters indicate a moderate degree of nucleotide conservation (>57% of the sequences contain this nucleotide). n, any nucleotide.

and in vivo studies have confirmed that Fnr binding sites in *E. coli* are partially symmetrical 22-bp sequences containing TTGA half-site motifs (18). Mutational analysis of the *E. coli* Fnr binding site has shown that the T in the first position of the half-site and the G in the third position are essential for Fnr function (6). Only one of the sequences listed in Table 2, that of *hemB* from *B. japonicum*, does not have a T in the first position of both half-sites; the G is 100% conserved among all of the sequences listed. In addition to lacking a T in the first position of one of the half-sites, the *B. japonicum hemB* Fnr box is located at a translational start site, making it unlikely to function in *cis* to activate *hemB*. The *B. japonicum hemH* gene, encoding ferrochelatase (16), is not listed in Table 4 because it contains only a half-site (TTGAT) in its promoter at position −150 relative to the translational start site. However, the half-site (TTGAT) centered at position −94 relative to the transcriptional start site in the *E. coli ndh* promoter has been shown to be functional (18). It is not yet known whether either *hemB* or *hemH* is regulated by oxygen.

The only Fnr box listed that has been shown to be functional besides that upstream of the *B. japonicum hemA* gene (this study) is that centered at position −487 upstream of the *R. meliloti fnr*-like gene *fixK* (58). Site-directed mutagenesis demonstrated that this site confers negative autoregulation on the *fixK* gene. A number of other genes listed in Table 4 have been shown to be regulated by *fixK* or *fnrN*, *fnr*-like genes. These include *fixN* from *R. meliloti* (8), *Azorhizobium caulinodans* (35), and *B. japonicum* (2) and *fnrN* from *R. leguminosarum* (49).

The presence of an Fnr box upstream of the *R. meliloti hemA* gene suggests that the *R. meliloti* gene, like the *B. japonicum* gene, is responsive to oxygen levels. Batut et al. (5) reported that an *R. meliloti hemA-lacZ* fusion was expressed microaerobically independently of the *R. meliloti fixK* gene (5). However, cells were exposed to lowered oxygen levels for only 2 h before β-galactosidase activity was measured. This may not have been enough time for induction of *hemA* expression. The levels of β-galactosidase activity reported for cells exposed for 2 h to microaerobiosis were apparently no different from those of aerobically grown cells. Of course, it is also possible that another, not yet identified *fnr*-like gene in *R. meliloti* controls *hemA* expression. Batut et al. did point out that the *R. meliloti fixK* gene is not the functional equivalent of *fnr* as defined in *E. coli* because it does not regulate nitrate reduction in *R. meliloti* (5), thus implying that there must be another *fnr*-like gene which does regulate nitrate respiration in *R. meliloti*.

Oxygen regulation of *hemA* is *fixLJ* dependent. We wished to determine if the regulation of *hemA* by oxygen involves the two-component FixLJ regulatory system that controls symbiotically important genes in *B. japonicum* in response to oxygen tension (1). *fixL* and *fixJ* mutant strains 7404 and 7361, respectively, were constructed by insertion of the *aph* gene, encoding kanamycin resistance, so that *aph* is transcribed in the opposite direction from *fixL* and *fixJ* (1). Plasmid pBJ289, carrying a *hemA-lacZ* fusion with 518 bp upstream of the *hemA* transcriptional start site, was conjugally transferred into strains 7404 and 7361. In cultures shifted from aerobic growth to conditions of restricted aeration, *hemA-lacZ* fusion activity is not induced (Fig. 2), implicating both *fixL* and *fixJ* in the regulation of *hemA* by oxygen. We verified that control of *hemA* through *fixLJ* is specific for oxygen by growing *fixL* and *fixJ* mutant strains containing the fusion under iron-deficient and iron-sufficient conditions (42). Activity of the *hemA-lacZ* fusion in these mutant strains mimicked that of wild-type strains carrying the fusion; activity was induced 3- and 4.5-fold in strains 7361 and 7404, respectively, although overall activity levels

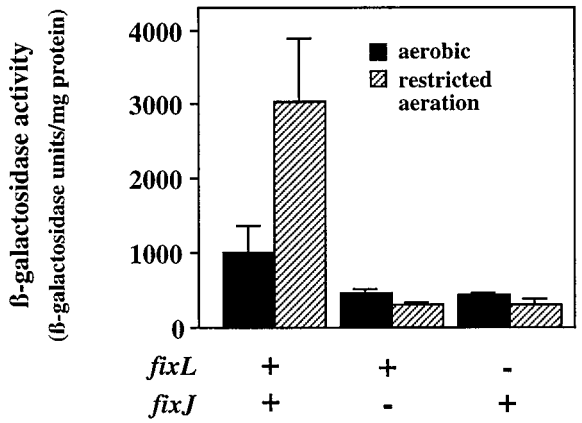

FIG. 2. Expression of the *B. japonicum hemA-lacZ* fusion in *fixL* and *fixJ* mutant strains of *B. japonicum* under conditions of restricted aeration. The *B. japonicum* mutant strains 7404 (*fixL*) and 7361 (*fixJ*) and *B. japonicum* wild-type strain 110 each contain plasmid pBJ289 carrying the *hemA-lacZ* fusion. Cultures of *B. japonicum* were frozen 48 h after transfer to restricted aeration for later assay of β-galactosidase activity as described in Materials and Methods.

were lower than that of wild-type strain 110 carrying the same fusion (Table 3).

Oxygen control of ALA synthesis in other organisms. Oxygen control of Δ-aminolevulinic acid (ALA) synthesis in *E. coli* (11), in the photosynthetic bacterium *Rhodobacter capsulatus* (22, 60), and in the baker's yeast *Saccharomyces cerevisiae* (27) has been documented. In *E. coli*, which synthesizes ALA via a pathway different from that of rhizobia, the *hemA* gene encodes glutamyl-tRNA dehydrogenase, which catalyzes the rate-limiting step in the production of ALA from glutamate. Its expression, like that of *hemA* in *B. japonicum*, responds to lowered oxygen levels; the activity of an *E. coli hemA-lacZ* fusion is 2.5-fold higher in cells grown anaerobically than in those grown aerobically (9). However, in an Fnr mutant strain of *E. coli*, *hemA-lacZ* activity is increased eightfold, indicating that Fnr represses *hemA* activity. This is opposite to our results, which indicate a positive role for an Fnr-like protein in regulating *hemA* expression. *hemA* expression in *E. coli* is positively regulated by ArcA under aerobic and anaerobic conditions (9). Thus, there are opposing controls on *hemA* expression in *E. coli* in response to lowered-oxygen tension. This dual control by Fnr and ArcA allows *hemA* expression to increase under microaerobic conditions, as it does in the case of *B. japonicum*. In *S. cerevisiae*, the *HEM13* gene encoding coproporphyrinogen oxidase, the sixth enzyme of the heme biosynthetic pathway, is induced under low oxygen levels (26). *HEM13* is one of the hypoxic genes in *S. cerevisiae*; products of hypoxic genes allow cells to utilize limiting oxygen supplies more efficiently. Other genes in the heme biosynthetic pathway, including *HEM1*, which encodes ALAS, are also responsive to oxygen, although their expression appears constitutive because of a balance of positive and negative controls (27). Opposing regulatory mechanisms may allow cells to transiently adjust their heme concentration following a shift to a new growth condition, such as the addition of a nonfermentable carbon source (27).

Implications for the symbiosis. We can now add *hemA* to the list of genes regulated by FixLJ. In *B. japonicum*, FixLJ activates genes required for anaerobic nitrate respiration and for microaerobic respiration in response to low-oxygen tension (2). This activation is proposed to occur via a regulatory cascade, with *fixJ* activating $fixK_2$, which then activates various target genes (2). In addition, $fixK_2$ regulates another *fnr*-like gene, $fixK_1$, whose targets are not yet known. Because microaerobic expression of the *B. japonicum hemA* gene is mediated via the *cis*-acting *hemA* Fnr box and the *trans*-acting FixLJ two-component regulatory system, we hypothesize that an *fnr*-like gene, possibly $fixK_2$, is controlling oxygen regulation of *hemA* in *B. japonicum* by binding to the Fnr box and activating gene transcription. Given the central role of FixLJ in coordinating symbiotic gene expression in rhizobia, it seems reasonable to conclude that heme biosynthesis in bacteroids is under symbiotic control. Although we cannot rule out the idea that FixJ itself could be regulating *hemA*, the requirement for the Fnr box for oxygen regulation lends support to the involvement of the Fnr homolog FixK. Waelkens et al. (58) have derived a potential FixJ consensus binding site from the promoters of some FixJ-regulated genes. There is no match to this consensus sequence in the *hemA* promoter. The fact that FixLJ regulates genes involved in nitrogen fixation strongly supports the importance of *B. japonicum hemA* expression in the symbiosis and suggests a correlation between heme synthesis and the expression of symbiotically important heme-containing proteins, such as the terminal oxidase encoded by *fixNOQP* (2).

hemA expression has now been monitored at the level of *hemA* mRNA abundance (61), activity of *hemA-lacZ* fusion constructs (references 41 and 42 and this study), and activity of the endogenous ALAS enzyme itself (e.g., references 19, 46, and 47). Because bacteroids have 17-fold more *hemA* mRNA than cultured cells (53) and yet show no increase in endogenous ALAS activity, it is clear that there must be some type of posttranscriptional control. Ongoing work in the laboratory has implicated heme in this posttranscriptional regulation of *hemA* expression (41). Thus, bacteroid ALAS expression in nodules is a composite of activation by low oxygen levels and repression by heme, similar to what is seen with the ALAS gene of *S. cerevisiae* (27).

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REFERENCES

1. **Anthamatten, D., and H. Hennecke.** 1991. The regulatory status of the *fixL*- and *fixJ*-like genes may be different from that in *Rhizobium meliloti*. Mol. Gen. Genet. **225:**38–48.
2. **Anthamatten, D., P. Rossi, B. Scherb, M. Babst, H. M. Fischer, and H. Hennecke.** 1994. Genetic control of nitrate respiration and microaerobic respiration in *Bradyrhizobium japonicum* by a complex regulatory cascade involving two FNR-like proteins. Bioengineering **10:**26.
3. **Anthamatten, D., B. Scherb, and H. Hennecke.** 1992. Characterization of a *fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* genes. J. Bacteriol. **174:**2111–2120.
4. **Appleby, C. A.** 1984. Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant Physiol. **35:**443–478.
5. **Batut, J., M. L. Daveran-Mingot, M. David, J. Jacobs, A. M. Garnerone, and D. Kahn.** 1989. *fixK*, a gene homologus with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J. **8:**1279–1286.
6. **Bell, A. I., J. A. Cole, and J. W. Busby.** 1990. Molecular genetic analysis of an FNR-dependent anaerobically inducible *Escherichia coli* promoter. Mol. Microbiol. **4:**1753–1763.
7. **Chauhan, S., and M. R. O'Brian.** 1993. *Bradyrhizobium japonicum* δ-aminolevulinic acid dehydratase is essential for symbiosis with soybean and contains a novel metal-binding domain. J. Bacteriol. **175:**7222–7227.
8. **Colonna-Romano, S., W. Arnold, A. Schlüter, P. Boistard, A. Pühler, and U. B. Priefer.** 1990. An Fnr-like protein encoded in *Rhizobium leguminosarum* biovar *viciae* shows structural and functional homology to *Rhizobium*

meliloti FixK. Mol. Gen. Genet. **223:**138–147.
9. **Darie, S., and R. P. Gunsalus.** 1994. Effect of heme and oxygen availability on *hemA* gene expression in *Escherichia coli*: role of the *fnr*, *arcA*, and *himA* gene products. J. Bacteriol. **176:**5270–5276.
10. **David, M., M.-L. Daveran, J. Batut, A. Dedieu, O. Domergue, J. Ghai, C. Hertig, P. Boistard, and D. Kahn.** 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell **54:**671–683.
11. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
12. **Drolet, M., and A. Sasarman.** 1991. Cloning and nucleotide sequence of the *hemA* gene of *Agrobacterium radiobacter*. Mol. Gen. Genet. **226:**250–256.
13. **Eiglmeier, K., N. Honore, S. Iuchi, E. C. C. Lin, and S. T. Cole.** 1989. Molecular genetic analysis of FNR-dependent promoters. Mol. Microbiol. **3:**869–878.
14. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
15. **Fischer, H.-M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
16. **Frustaci, J. M., and M. R. O'Brian.** 1992. Characterization of a *Bradyrhizobium japonicum* ferrochelatase mutant and isolation of the *hemH* gene. J. Bacteriol. **174:**4223–4229.
17. **Gilles-Gonzalez, M. A., G. S. Ditta, and D. R. Helinski.** 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. Nature (London) **350:**170–172.
18. **Green, J., and J. R. Guest.** 1994. Regulation of transcription at the *ndh* promoter of *Escherichia coli* by FNR and novel factors. Mol. Microbiol. **12:**433–444.
19. **Guerinot, M. L., and B. K. Chelm.** 1986. Bacterial aminolevulinic acid synthase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhizobium japonicum* symbiosis. Proc. Natl. Acad. Sci. USA **83:**1837–1841.
20. **Guerinot, M. L., B. A. Morisseau, and T. Klapatch.** 1990. Electroporation of *Bradyrhizobium japonicum*. Mol. Gen. Genet. **221:**287–290.
21. **Gunsalus, R. P.** 1992. Control of electron flow in *Escherichia coli*: coordinated transcription of respiratory pathway genes. J. Bacteriol. **174:**7069–7074.
22. **Hornberger, U., B. Wieseler, and G. Drews.** 1991. Oxygen tension regulated expression of the *hemA* gene of *Rhodobacter capsulatus*. Arch. Microbiol. **156:**129–134.
23. **Hunt, S., and D. B. Layzell.** 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44:**483–511.
24. **Kahn, D., M. David, O. Domergue, M. L. Daveran, J. Ghai, P. R. Hirsch, and J. Batut.** 1989. *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J. Bacteriol. **171:**929–939.
25. **Kaminski, P. A., and C. Elmerich.** 1991. Involvement of *fixLJ* in the regulation of nitrogen fixation in *Azorhizobium caulinodans*. Mol. Microbiol. **5:**665–673.
26. **Keng, T.** 1992. *HAP1* and *ROX1* form a regulatory pathway in the repression of *HEM13* transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**2616–2623.
27. **Keng, T., and L. Guarente.** 1987. Constitutive expression of the yeast *HEM1* gene is actually a composite of activation and repression. Proc. Natl. Acad. Sci. USA **84:**9113–9117.
28. **Kim, H., and R. J. Maier.** 1990. Transcriptional regulation of hydrogenase synthesis by nickel in *Bradyrhizobium japonicum*. J. Biol. Chem. **265:**18729–18732.
29. **Kim, H., C. Yu, and R. J. Maier.** 1991. Common *cis*-acting region responsible for transcriptional regulation of *Bradyrhizobium japonicum* hydrogenase by nickel, oxygen, and hydrogen. J. Bacteriol. **173:**3993–3999.
30. **Kullik, I., S. Fritsche, H. Knobel, J. Sanjuan, H. Hennecke, and H.-M. Fischer.** 1991. *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the σ^{54} gene (*rpoN*). J. Bacteriol. **173:**1125–1138.
31. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
32. **Legocki, R. P., A. C. Yun, and A. A. Szalay.** 1984. Expression of β-galactosidase controlled by a nitrogenase promoter in stem nodules of *Aeschynomene scabra*. Proc. Natl. Acad. Sci. USA **81:**5806–5810.
33. **Leong, S. A., P. H. Williams, and G. S. Ditta.** 1985. Analysis of the 5′ regulatory region of the gene for aminolevulinic acid synthetase of *Rhizobium meliloti*. Nucleic Acids Res. **13:**5965–5976.
34. **Lois, A. F., M. Weinstein, G. S. Ditta, and D. R. Helinski.** 1993. Autophosphorylation and phosphatase activities of the oxygen-sensing protein FixL of *Rhizobium meliloti* are coordinately regulated by oxygen. J. Biol. Chem. **268:**4370–4375.
35. **Mandon, K., P. A. Kaminski, and C. Emerich.** 1994. Functional analysis of the *fixNOPQ* region of *Azorhizobium caulinodans*. J. Bacteriol. **176:**2560–2568.
36. **McClung, C. R., J. E. Somerville, M. L. Guerinot, and B. K. Chelm.** 1987. Structure of the *Bradyrhizobium japonicum* gene *hemA* encoding 5-aminolevulinic acid synthase. Gene **54:**133–139.
37. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Nees, D. W., P. A. Stein, and R. A. Ludwig.** 1988. The *Azorhizobium caulinodans nifA* gene: identification of upstream-activating sequences including a new element, the "anaerobox." Nucleic Acids Res. **16:**9839–9853.
39. **Neidle, E. L., and S. Kaplan.** 1993. Expression of the *Rhodobacter sphaeroides hemA* and *hemT* genes, encoding two 5-aminolevulinic acid synthase isozymes. J. Bacteriol. **175:**2292–2303.
40. **O'Brian, M. R., and R. J. Maier.** 1989. Molecular aspects of the energetics of nitrogen fixation in *Rhizobium*-legume symbioses. Biochim. Biophys. Acta **974:**229–246.
41. **Page, K. M.** 1994. The effect of iron, oxygen and heme on the expression of the *Bradyrhizobium japonicum hemA* gene. Ph.D. thesis. Dartmouth College, Hanover, N.H.
42. **Page, K. M., E. L. Connolly, and M. L. Guerinot.** 1994. The effect of iron availability on expression of the *Bradyrhizobium japonicum hemA* gene. J. Bacteriol. **176:**1535–1538.
43. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. USA **90:**3309–3313.
44. **Ratet, P., K. Pawlowski, J. Schell, and F. J. de Bruijn.** 1989. The *Azorhizobium caulinodans* nitrogen-fixation regulatory gene *nifA* is controlled by the cellular nitrogen and oxygen status. Mol. Microbiol. **3:**825–838.
45. **Reyrat, J.-M., M. David, C. Blonski, P. Boistard, and J. Batut.** 1993. Oxygen-regulated in vitro transcription of *Rhizobium meliloti nifA* and *fixK* genes. J. Bacteriol. **175:**6867–6872.
46. **Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. Keyser.** 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybeans. Appl. Environ. Microbiol. **53:**2624–2630.
47. **Sangwan, I., and M. R. O'Brian.** 1991. Evidence for an inter-organismic heme biosynthetic pathway in symbiotic soybean root nodules. Science **251:**1220–1222.
48. **Sangwan, I., and M. R. O'Brian.** 1992. Characterization of Δ-aminolevulinic acid formation in soybean root nodules. Plant Physiol. **98:**1074–1079.
49. **Schlüter, A., T. Patschkowski, G. Unden, and U. B. Priefer.** 1992. The *Rhizobium leguminosarum* FnrN protein is functionally similar to *Escherichia coli* Fnr and promotes heterologous oxygen-dependent activation of transcription. Mol. Microbiol. **6:**3395–3404.
50. **Schueddekopf, K., S. Hennecke, M. Kutsche, U. Liese, and W. Klipp.** 1993. Characterization of *anf* genes specific for the alternative nitrogenase and identification of *nif* genes required for both nitrogenases in *Rhodobacter capsulatus*. Mol. Microbiol. **8:**673–684.
51. **Shapleigh, J. P., and R. B. Gennis.** 1992. Cloning, sequencing, and deletion from the chromosome of the gene encoding subunit 1 of the aa3 type cytochrome oxidase of *Rhodobacter sphaeroides*. Mol. Microbiol. **6:**635–642.
52. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single- and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
53. **Somerville, J. E., and B. K. Chelm.** 1988. Regulation of heme biosynthesis in *Bradyrhizobium japonicum*, p. 111–112. *In* R. Palacios and D. P. S. Verma (ed.), Molecular genetics of plant-microbe interactions. APS Press, St. Paul, Minn.
54. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek **66:**23–36.
55. **Spiro, S., and J. R. Guest.** 1990. Fnr and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Rev. **75:**399–428.
56. **Squartini, A., R. J. Van Veen, T. Regensberg-Tuink, P. J.-J. Hooykaas, and M. P. Nuti.** 1988. Identification and characterization of the *nodD* gene in *Rhizobium leguminosarum* 1001. Mol. Plant-Microbe Interact. **1:**145–149.
57. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. glycinea. J. Bacteriol. **169:**5789–5794.
58. **Waelkens, F., A. Foglia, J. B. Morel, J. Fourment, J. Batut, and P. Boistard.** 1992. Molecular genetic analysis of the *Rhizobium meliloti fixK* promoter: identification of sequences involved in positive and negative regulation. Mol. Microbiol. **6:**1447–1456.
59. **Weinstein, M., A. F. Lois, G. S. Ditta, and D. R. Helinski.** 1993. Mutants of the two-component regulatory protein FixJ of *Rhizobium meliloti* that have increased activity at the *nifA* promoter. Gene **134:**145–152.
60. **Weinstein, M., A. F. Lois, E. K. Monson, G. S. Ditta, and D. R. Helinski.** 1992. Isolation of phosphorylation-deficient mutants of the *Rhizobium meliloti* two-component regulatory protein, FixJ. Mol. Microbiol. **6:**2041–2049.
61. **Wright, M. S., J. J. Eckert, S. W. Biel, and A. J. Biel.** 1991. Use of a *lacZ* fusion to study transcriptional regulation of the *Rhodobacter capsulatus hemA* gene. FEMS Microbiol. Lett. **78:**339–342.